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Enhanced induction of a histamine-forming enzyme, histidine decarboxylase, in mice primed with NOD1 or NOD2 ligand in response to various Toll-like receptor agonists

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We investigated the immunopharmacological aspects of innate immune responses via Toll-like receptors (TLRs), NOD1 and NOD2, in terms of induction of the histamine-forming enzyme, histidine decarboxylase (HDC), activity in mice. Intravenous injection of TLR4-agonistic synthetic lipid A definitely induced HDC activity in the liver, spleen, and lungs, especially the lungs, in mice, where maximum activity was induced about 3 h after the injection of lipid A. The TLR2/6 agonistic synthetic diacyl-type lipopeptide FSL-1 and TLR3-agonistic poly I:C were also effective in inducing HDC, while the NOD2-agonistic synthetic muramyldipeptide (MDP) and NOD1-agonistic synthetic FK156 and FK565 exhibited only weak activities in this respect. Mice primed with intravenous injection of NOD1 or NOD2 agonists produced higher HDC activity following the 4–6 h later intravenous challenge with the above TLR agonists. Among the priming agents, FK565 exhibited the strongest activity, and it was effective via various administration routes – intraperitoneal, subcutaneous, intramuscular, as well as intravenous injection; furthermore, oral (gastric) administration was effective, although it needed a dose 10 times higher than that required for other administration routes. These findings suggest that HDC is induced in association with TLRs and NOD1/2, and that the newly formed histamine by the induced HDC might play important roles in the regulation of inflammatory and immune responses in various organs.

Keywords: histamine, Toll-like receptors, NOD1, NOD2, lipid A

Introduction

Peptidoglycans (PGNs) are ubiquitous constituents of bacterial cell walls, and are distributed in both Gram-positive and Gram-negative bacteria, except *Mycoplasma*, which lack cell walls. Peptidoglycan exhibited various immunobiological activities.[1] In 2003, two nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family molecules were revealed to be intracellular receptors for PGN: NOD1 and NOD2 recognize *meso*-diaminopimelic acid (*meso*-DAP)-containing peptide moiety[2,3] and muramyldipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine) moiety,[4,5] respectively; therefore, NLRs are important pattern-recognition molecules (PRMs) which recognize microbe-associated molecular patterns (MAMPs) in innate immunity. On the other hand, Toll-like receptor (TLR) family molecules are well-characterized PRMs which recognize various MAMPs.[6,7] Endotoxic lipopolysaccharide (LPS) is ubiquitously distributed in the outer membrane of Gram-negative bacteria and its active entity is lipid A, which is recognized by TLR4. Lipoproteins exist in the cytoplasmic membrane of almost all bacterial species, including *Mycoplasma*,

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whose active entity is lipopeptide, which is mainly recognized by TLR2. Furthermore, *Mycoplasma*-type lipopeptide is di-acylated and recognized by TLR2 and TLR6.[8] Viral RNA motif is also recognized by TLR molecules: double-stranded RNA is recognized by TLR3.

Histamine is a typical mediator of inflammation and allergy. This vasoactive amine modulates various immune responses, including modulation of the T-helper 1 (Th1)–Th2 balance and possibly hematopoiesis.[9] It is well known that histamine is not only released and stored in mast cells or basophiles, but also supplied to various tissues through induction of the histamine-forming enzyme, histidine decarboxylase (HDC). Indeed, injection into mice of interleukin (IL)-1 and tumor necrosis factor (TNF)-α induces HDC in various tissues, such as the liver, lungs, spleen and bone marrow.[10] On the other hand, hematopoietic cytokines, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF), only induce HDC in hematopoietic organs, *i.e*. bone marrow and spleen.[11] Wu *et al*.[12] reported that LPS and IL-1 induced HDC in various tissues in a mast cell-independent manner. The major cells in which HDC is induced in non-haematopoietic organs (*i.e*. liver and lungs) and hematopoietic organs (*i.e*. spleen and bone marrow) are supposed to be vascular endothelial cells and hematopoietic precursor cells, respectively.[13]

The priming effects of NOD1 and NOD2 agonists to augment responses against TLR agonists have so far been investigated in terms of cytokine production.[14–16] In this study, we examined whether various TLR agonists and NOD1 and NOD2 agonists induce HDC activity in various tissues. Furthermore, we examined the possible priming activity of NOD1 and NOD2 agonists to augment the induction of HDC activity in response to various TLR agonists in mice. The induction of high levels of HDC may induce high levels of histamine, which in turn may induce inflammatory responses and modulate immune responses in relation to various microbial infections, including commensal microbes.

Materials and Methods

Mice

Male BALB/c mice (6–7 weeks old) were obtained from the facility for experimental animals in Tohoku University. All experiments complied with the Guidelines for the Care and Use of Laboratory Animals of Tohoku University.

Reagents

In this study, we mainly used chemically synthesized reagents to avoid the influence of minor components in bacterial fractions that could affect the results. The *Mycoplasma*-type diacyl lipopeptide FSL-1 (synthetic TLR2/6 agonist)[17] was purchased from EMC microcollections (Tübingen, Germany). Double-stranded RNA poly I:C (TLR3 agonist) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). A synthetic *Escherichia coli*-type lipid A (LA-15-PP, TLR4 agonist) and synthetic MDP (NOD2 agonist) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). The synthetic NOD1 agonist, FK156[18] and its derivative, FK565,[18] were kindly supplied by Astellas Pharmaceutical Inc. (Tokyo, Japan). The FK156 was chemically synthesized to mimic an active compound found in fermentation broths of strains of *Streptomyces*, and FK565 was the leading compound among various FK156 derivatives. The chemical structures of these synthetic ligands are shown in Figure 1. All reagents were dissolved in sterile saline and administered to mice.

Assay of HDC activity

After mice were decapitated, their lungs, liver and spleen were rapidly removed before being frozen in a box containing dry ice and stored at -80°C until assayed for HDC activity. In each tissue, HDC activity was assayed using a previously described method and expressed in nmol of histamine formed per hour per gram of tissue (nmol/h/g).[19,20]

Statistical analysis

Experimental values are given as the mean $\pm$ SD. The statistical significance of differences was analyzed using Student's unpaired *t*-test. *P*-values less than 0.05 were significant.

Results

Priming effects of NOD1 ligands, FK156 and FK565, to augment HDC activity induction in lungs in response to lipid A

Throughout this study, we used primarily synthetic ligands mimicking microbial MAMPs to avoid the influence of bioactive minor components in microbial fractions.

First, we examined the possible priming effects of NOD1 agonists, FK156 and FK565. Before the

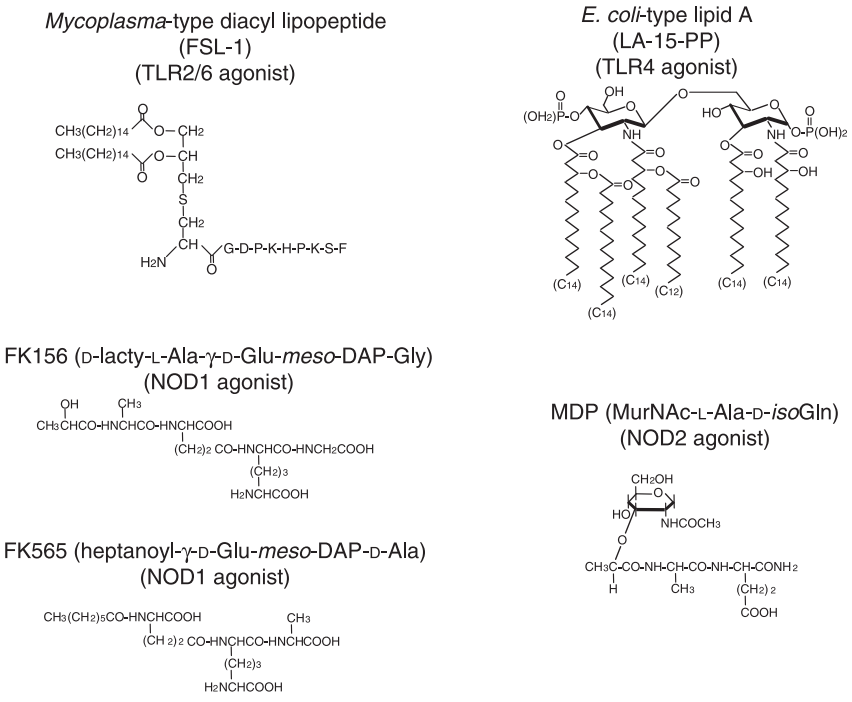

Fig. 1. Chemical structures of synthetic ligands. Chemical structures of *Mycoplasma*-type diacyl lipopeptide FSL-1 (TLR2/6 agonist), *E. coli*-type lipid A (LA-15-PP, TLR4 agonist), FK156 (D-lactyl-L-Ala-Γ-D-Glu-*meso*-DAP-Gly, NOD1 agonist) and its derivative FK565 (heptanoyl-γ-D-Glu-*meso*-DAP-D-Ala, NOD1 agonist) and muramyldipeptide (MDP; MurNAc-L-Ala-D-*iso*Gln, NOD2 agonist) are shown.

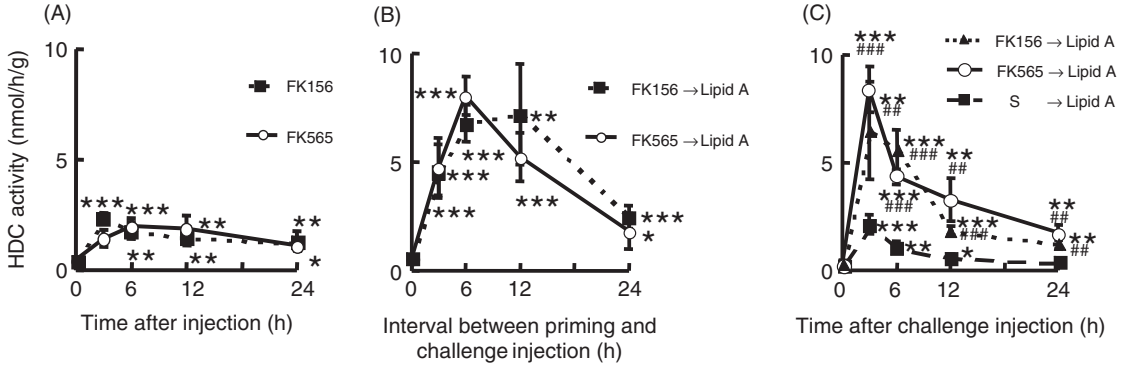

Fig. 2. Priming effects of FK156 and FK565 to augment HDC activity in lungs in response to synthetic lipid A. (A) Mice (4 per group) were injected intravenously with FK156 or FK565 (5 mg/kg). The mice were killed to remove lungs at the times indicated to determine HDC activity. (B) Mice (4 per group) were injected intravenously with FK156 or FK565 (each 5 mg/kg); thereafter, at the various intervals indicated, mice were injected intravenously with synthetic lipid A (0.02 mg/kg). Three hours after the challenge injection, the mice were killed to remove lungs to determine HDC activity. (C) Mice (4 per group) were primed intravenously with saline (S), FK156 or FK565 (each 5 mg/kg), and 6 h later were challenged intravenously with synthetic lipid A (0.02 mg/kg). The mice were killed at the times indicated after the challenge injection to remove lungs to determine HDC activity. Each value is shown as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus time 0. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus respective lipid A alone control.

experiments, we examined whether NOD1 ligands themselves induce HDC activity in mice. As shown in Figure 2A, FK156 and FK565 induced significant and continuous HDC activity in the lungs, although the levels were low. With the aim of examining the priming effects of NOD1 agonists, mice were injected intravenously with NOD1 agonists; thereafter, at various intervals, the mice received intravenous challenge injection with synthetic lipid A. Three hours after the challenge injection, the lungs were removed to assay HDC activity. The tendency of priming effects of FK156 and FK565 in combination with lipid A was similar, and definite priming activity was observed at 3–12-h intervals between the priming and challenge injection, and the maximum priming effect was observed at around the 6-h interval (Fig. 2B). In a

separate experiment, optimum priming was observed at the 4–6-h interval (data not shown). When lipid A was challenged after this interval, maximum induction of HDC activity was observed 3 h after the challenge injection (Fig. 2C). We then examined the dose dependency of the priming activity of FK156 and FK565. FK565 exhibited significant priming activity at 10 μg/kg or 100 μg/kg (Fig. 3); however, 5 mg/kg FK156 was required to exhibit a significant priming effect (data not shown).

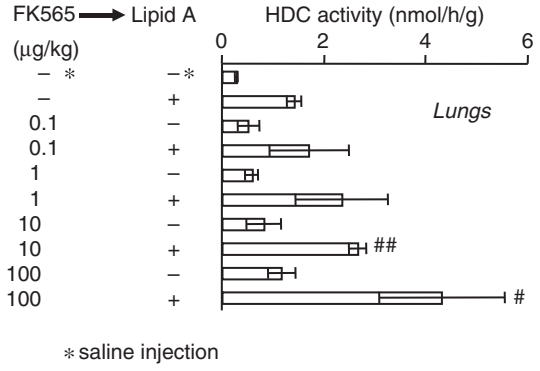

Fig. 3. Priming effects of FK565 to augment HDC activity in response to synthetic lipid A. Mice (4 per group) were injected intravenously with saline (S) or FK565 (0.1–100 μg/kg), and 6 h later were challenged intravenously with saline (S) or lipid A (0.02 mg/kg). Three hours after the challenge injection, the mice were killed to remove lungs to determine HDC activity. Each value is shown as the mean ± SD. $^{\#}P<0.05$, $^{\#\#}P<0.01$, S → lipid A.

Priming activity of FK565 administered via various routes

Next, we examined the priming effects of FK565 when it was administered by various administration routes. Mice were administrated with FK565 at 0.5 mg/kg, except in the case of *per os* (po, *i.e.* intra-gastric). As shown in Figure 4, intraperitoneal (ip), subcutaneous (sc) and intramuscular (im) as well as intravenous (iv) injection of FK565 (0.5 mg/kg) significantly augmented the HDC activity induction in response to synthetic lipid A. The FK565, when administered po, showed a significant priming effect at 5 μg/kg, but not at 0.5 μg/kg (data not shown).

Priming effects of a NOD2 ligand, MDP, to augment HDC activity induction in response to lipid A

As described above, pretreatment of mice with MDP induces endotoxin hypersensitivity accompanied by hyper-production of inflammatory cytokines.[15] Thus, we tested the priming effect of MDP in the induction of HDC activity by lipid A. As shown in Figure 5, MDP alone (5 mg/kg) induced HDC activity in various organs, and the levels and time course of HDC induction were similar to those shown for FK156 and FK565 (Fig. 2A); therefore, we designed the following experiments. Mice were injected into a tail vein with saline or MDP (5 mg/kg); 4.5 h later, mice were challenged

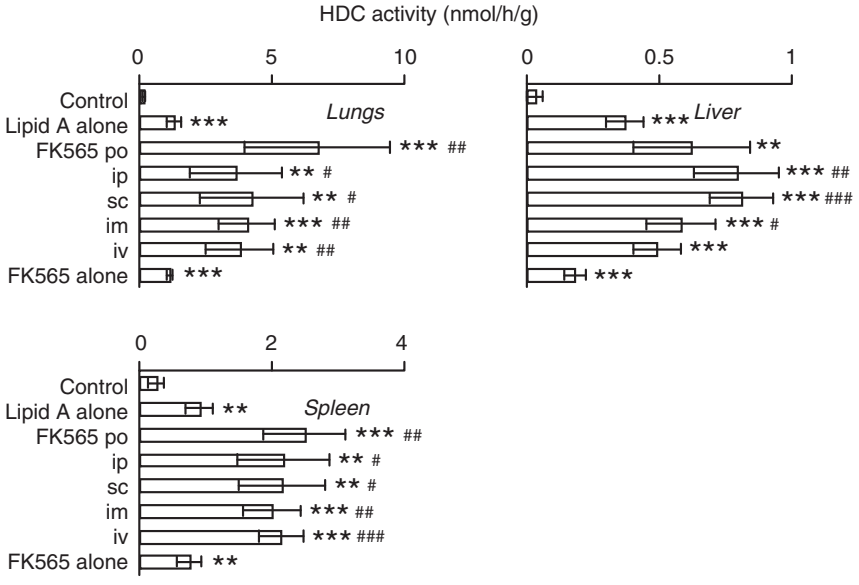

Fig. 4. Comparison of priming effects of FK565 in various administration routes in response to synthetic lipid A in mice. BALB/c mice (4 per group) were administrated *per os* (po), intraperitoneally (ip), subcutaneously (sc), intramuscularly (im) or intravenously (iv) with FK565 (0.5 mg/kg except po; 5 mg/kg in the case of po). After 6 h, the mice were injected intravenously with synthetic lipid A (0.02 mg/kg). Three hours after the second injection, the mice were killed to remove lungs, liver and spleen to determine HDC activity in the organs. Each value is shown as the mean ± SD from 4 mice. $^{**}P<0.01$, $^{***}P<0.001$ versus control. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus lipid A alone.

intravenously with saline or lipid A (0.02 mg/kg). Three hours after the challenge injection, the mice were killed by decapitation to remove the lungs, liver and spleen to determine HDC activity in the tissues. Although MDP alone induced only low levels of HDC activity, MDP-primed mice exhibited higher induction of HDC activity in the lungs, liver and spleen, in response to synthetic lipid A, than non-primed mice (Fig. 2). In particular, in the lungs and spleen, MDP exhibited marked priming activity to augment HDC activity in response to lipid A.

Priming effects of MDP, FK156 and FK565 to augment HDC activity induction in response to other TLR agonists

We first used lipid A (TLR4 ligand) as the challenging agent and then examined other TLR agonists. First, we tested a TLR2/6 agent, *Mycoplasma*-type synthetic diacyl lipopeptide FSL-1. Mice were injected intravenously with saline, MDP, FK156 or FK565 (each 5 mg/kg), and 4.5 h later the mice received intravenous challenge of FSL-1 (0.5 mg/kg). Three hours after the challenge injection, the mice were killed to remove the lungs, liver and spleen to determine HDC activity. Although FSL-1 alone significantly induced HDC activity in the organs, significantly higher levels of HDC activity were induced in the FK156-primed spleen and FK565-primed lungs and spleen than in non-primed mice, although no clear priming effect was observed in other cases (Fig. 6). Next, we examined a TLR3 agonist, poly I:C (double-stranded RNA). Mice were injected intravenously with saline or one of three NOD agonists (each 5 mg/kg), and 4.5 h later the mice were challenged intravenously with poly I:C (5 mg/kg). The poly I:C alone induced significant HDC activity in the liver and spleen (Fig. 7). In the lungs, MDP, FK156 and FK565 exhibited remarkable priming effects, although no clear priming effect was observed in other organs (Fig. 7).

DISCUSSION

In this study, we demonstrated that NOD1 and NOD2 agonists, especially NOD1-agonistic FK565, exhibited priming effects in mice to induce higher HDC activity in various organs upon stimulation with various TLR agonists than in non-primed mice. In 1987, Takada and Galsons[21] reported that pretreatment of mice with MDP enhanced the lethal toxicity of endotoxin. In the experiments, maximum priming activity was observed when LPS was administered 4–6 h after MDP-priming. Simultaneous administration with LPS and MDP was also effective, but LPS administrated before MDP was scarcely effective. This was the first report on the priming effect of MDP. Parant *et al.*[14] then showed that pretreatment of mice with MDP induced hyper-production of serum tumor necrosis factor-α (TNF-α) in response to endotoxic LPS.

Nagao *et al.*[22] reported the species dependency of *in vitro* macrophage activation of MDP; guinea pig and

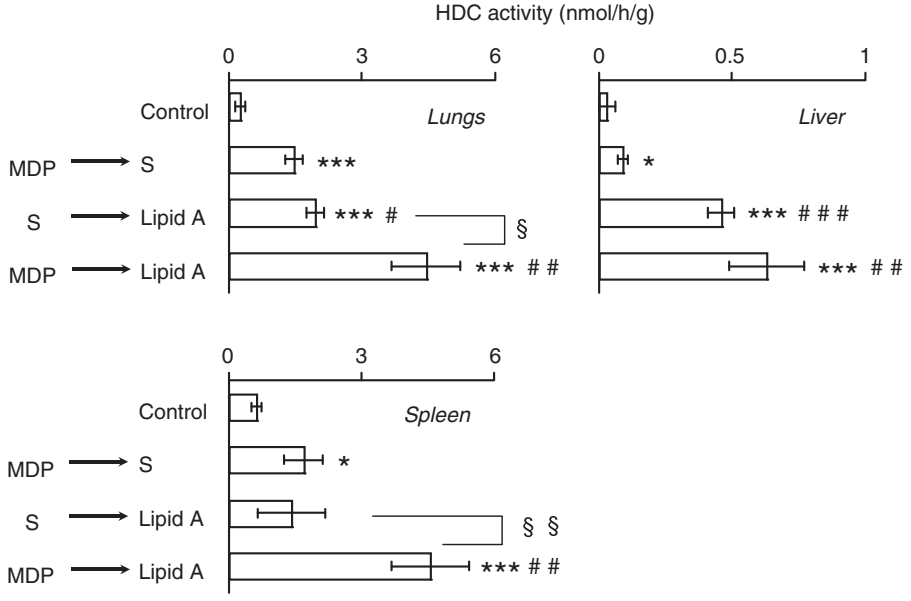

Fig. 5. Priming effect of MDP to augment HDC activity in response to synthetic lipid A. Mice (4 per group) were injected intravenously with saline (S) or MDP (5 mg/kg), and 4.5 h later were injected intravenously with S or lipid A (0.02 mg/kg). Three hours after the second injection, the mice were killed by decapitation to remove lungs, liver and spleen to determine HDC activity in the tissues. Each value is shown as the mean ± SD. $^{*}P<0.05$, $^{***}P<0.001$ versus control. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus MDP → S. $^{\S}P<0.05$, $^{\S\S}P<0.01$, between the two groups indicated.

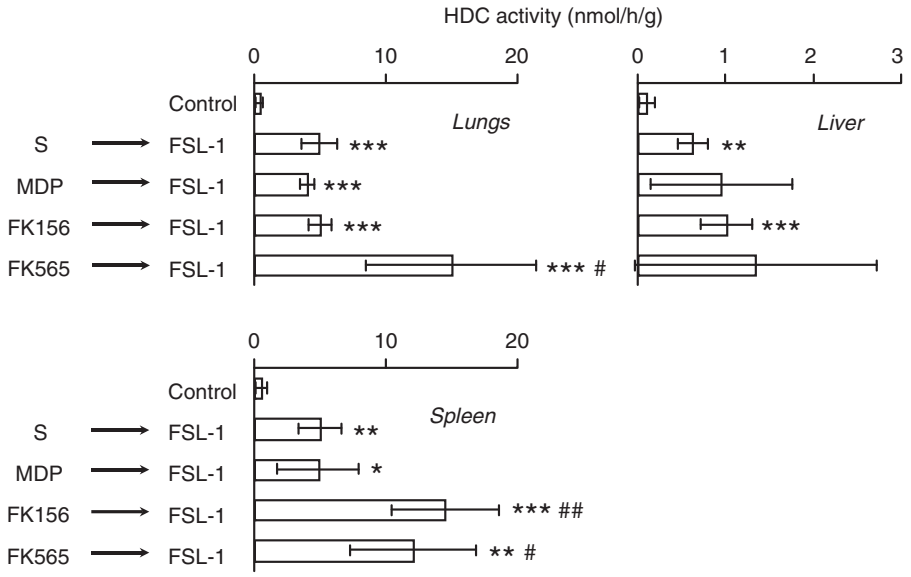

Fig. 6. Priming effects of MDP, FK156 and FK565 to augment HDC activity induction in response to FSL-1 in mice. BALB/c mice (4 per group) were injected intravenously with saline (S), MDP, FK156 or FK565 (each 5 mg/kg), and 4.5 h later were challenged intravenously with FSL-1 (0.5 mg/kg). Three hours after the challenge injection, the mice were killed to remove lungs, liver and spleen to determine HDC activity. Each value is shown as the mean ± SD from 4 mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus S → FSL-1.

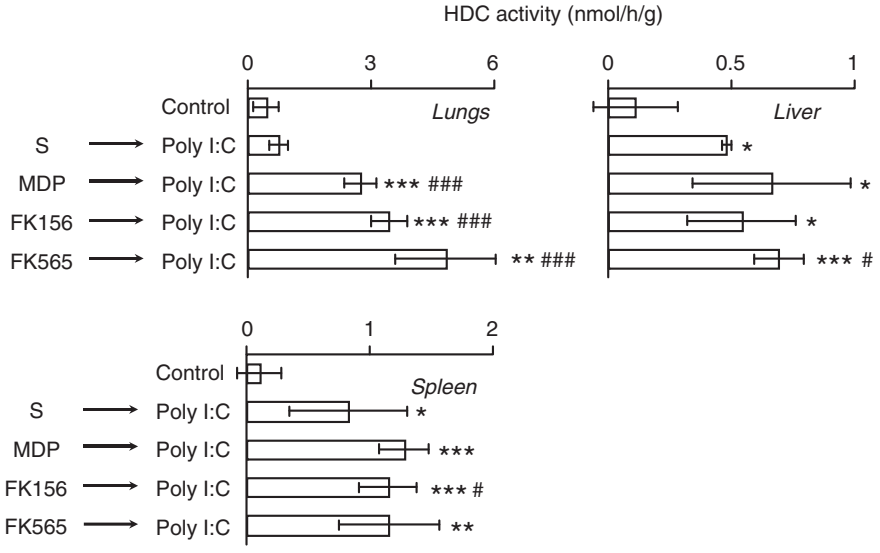

Fig. 7. Priming effects of MDP, FK156 and FK565 to augment HDC activity induction in response to poly I:C in mice. BALB/c mice (4 per group) were injected intravenously with saline (S), MDP, FK156 or FK565 (each 5 mg/kg), and 4.5 h later were challenged intravenously with poly I:C (0.5 mg/kg). Three hours after the challenge injection, the mice were killed to remove lungs, liver and spleen to determine HDC activity. Each value is shown as the mean ± SD from 4 mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control. $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ versus S → poly I:C.

rat cells were responsive, but murine cells were not. Regarding *in vivo* studies, adjuvant activity, especially that for cell-mediated immunity of MDP, has been examined mainly in guinea pigs. Furthermore, in guinea pigs, unlike mice, MDP alone exhibited a strong ability to induce inflammatory cytokines, but MDP-primed guinea pigs scarcely produced inflammatory cytokines in response to LPS, probably because of cytokine depletion.[23] In the present study, the NOD1 agonistic FK565 showed more powerful priming effects than the NOD2 agonistic MDP. We also observed the higher priming-activity of FK565 than MDP in mice to enhance inflammatory cytokine production in response to endotoxin.[16] In contrast, Uehara *et al*.[24] reported that MDP showed more powerful synergistic activation than FK156 and FK565 in human monocytic THP-1 cells.

Mice, in contrast to humans, may be low-responders to NOD2 stimulation and high-responders to NOD1 stimulation.

As described above, NOD1 and NOD2 agonists in combination with TLR agonists synergistically induced inflammatory cytokines in human monocytic cells in culture. Yang *et al*.[25] first demonstrated the marked synergistic effect of MDP and LPS to induce IL-8 and TNF-α in human monocytic THP-1 and U937 cells in culture. They also reported that THP-1 cells primed with MDP exhibited enhanced production of IL-8 upon later stimulation with LPS, while the reverse was not effective (*i.e*. LPS priming followed by MDP stimulation). In MDP-primed THP-1 cells, mRNA expression of MyD88, an adapter molecule for various TLRs, was definitely up-regulated, which might be partially responsible for the priming and synergistic effects. Uehara *et al*.[24] demonstrated that various chemically synthesized TLR agonists, which were also used in this study, in combination with NOD1 and NOD2 agonists synergistically activated nuclear factor (NF)-κB in THP-1 cells, resulting in synergistic induction of IL-8. In a murine *in vivo* study, synergistic phosphorylation of IκBα/β was observed in the liver of mice primed with NOD1 or NOD2 agonist followed by LPS stimulation (Hagiwara *et al*., unpublished observations). Similar synergism might be involved in HDC induction, and further studies are required to elucidate the mechanism of cross-talk between NOD1/2 and various TLRs.

Histidine decarboxylase is induced in various tissues in response to various cytokines and microbial ligands. The main producers of HDC in organs, such as the liver and lungs, are thought to be vascular endothelial cells, while in hematopoietic organs, such as the bone marrow and spleen, myeloid precursor cells might be major HDC producers. When host cells encounter bacteria, they should receive simultaneous stimulation through two pathways – NOD1 and/or NOD2 stimulation by PGN fragments and TLR stimulation by bacterial components, such as endotoxin and lipopeptide. In the case of hyper-infection by viruses and bacteria, additional TLR stimulation should occur. As shown in this study, combined NOD1/NOD2 and TLR stimulation powerfully induced HDC activity, resulting in hyper-production of histamine. Considering the multiple functions of histamine, 'neo-histamine', which is newly formed by the induced HDC, possibly plays important roles in increasing permeability in microcirculation, and in regulation of inflammatory and immune responses; thus, it might be important contributor to innate immune responses.

Peptidoglycan is a ubiquitous structure on the bacterial cell surface, whose active moieties possibly activate NOD2 and/or NOD1. Symbiosis with various bacteria or microbes is inevitable for mammals, including humans; therefore, mammals receive continuous NOD2 and/or NOD1 stimulation. In other words, mammals may have been naturally primed with NOD2 and/or NOD1; therefore, innate and adaptive immune responses are induced based on NOD1/2 priming. These possibilities could be investigated using NOD1 and NOD2 double knockout mice or RIP-2[26] knockout mice.

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